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Research Article

Invasive species monitoring based on eDNA multiplex PCR sequencing

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Abstract

Invasive species pose a significant threat to global biodiversity and the stability of ecosystems. Although environmental DNA (eDNA)-based quantitative PCR is considered effective, its limited multiplexing capacity makes it impractical for large-scale monitoring of invasive species. To address this limitation, we developed a novel and efficient approach for invasive species monitoring by combining multiplex PCR amplification with high-throughput sequencing. In this study, we screened 46 aquatic invasive species of major concern in China. We have integrated and designed 91 pairs of primers that can simultaneously amplify these species in a single PCR system. The validated method was applied to field monitoring in the Pearl River Basin to evaluate its practical performance. Multiplex PCR sequencing successfully detected 28 invasive species, with over 90% of environmental samples testing positive for invasive species DNA, demonstrating the method's high sensitivity and broad applicability. Furthermore, all 11 invasive species identified through metabarcoding were also consistently detected by multiplex PCR sequencing, showing a strong positive correlation and high concordance across all monitoring sites. In conclusion, multiplex PCR sequencing represents a powerful and cost-effective tool for simultaneously detecting multiple aquatic invasive species in the early stages of invasion. It significantly improves detection efficiency, reduces monitoring costs and provides a solid foundation for developing a scientific and scalable monitoring system for aquatic invasive species.

Key words: Amplicon sequencing, biodiversity monitoring, estuary ecosystem, metabarcoding, tilapia

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Introduction

Invasive Alien Species (IAS) are defined as non-native organisms capable of forming self-sustaining populations in new ecosystems, often leading to substantial ecological disruption, economic losses or societal challenges (Early et al. 2016; Pysek et al. 2020). As globalisation accelerates and human activities intensify, the frequency of species transport and introduction has

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increased, leading to a rise in the occurrence of IAS (Havel et al. 2015). IAS are especially notable for their high adaptability and reproductive capacity, which are facilitated by the open and connected nature of aquatic ecosystems. These characteristics enable them to rapidly establish, spread and alter local ecological dynamics within a short timeframe (Jerde et al. 2011; Abdo et al. 2013; Ray and Umapathy 2022). For example, *Eichhornia crassipes* (water hyacinth), a floating plant native to South America, often forms a dense floating layer that blocks sunlight and reduces oxygen levels in the water, inhibiting the growth of submerged vegetation and ultimately disrupting the ecological balance of freshwater ecosystems (Wilson et al. 2005).

Currently, species monitoring primarily relies on physical identification methods, including visual surveys and the use of fishing nets. While these methods are simple, intuitive and reliable (Thomsen and Willerslev 2015), they have several limitations, including a cumbersome monitoring process, time consumption, low species resolution, poor accuracy and high costs (Shen et al. 2016). Traditional techniques, such as electrofishing and netting, can also have environmental impacts, especially in freshwater ecosystems (Lundberg et al. 2000; Relyea 2005). Consequently, there is a pressing need for more efficient and environmentally friendly monitoring technologies.

In recent years, environmental DNA (eDNA) technology has emerged as a highly sensitive method for detecting aquatic species by collecting DNA fragments from water or soil. Amongst these, metabarcoding has become a focal point in ecological research, utilising high-throughput DNA sequencing for comprehensive monitoring of species. Olds et al. (2016) compared the effectiveness of electrofishing and eDNA metabarcoding in American streams and demonstrated that eDNA can detect more fish species than traditional methods, highlighting its unique advantages in species monitoring. Additionally, metabarcoding can be used as a monitoring tool to screen for invasive species; however, it has limitations, such as reduced sensitivity for rare or low-abundance taxa and the potential for non-target amplification. These drawbacks have prompted the development of species-specific assays, such as qPCR and ddPCR, which offer higher sensitivity and specificity (Thomas et al. 2019; Feng et al. 2023; Nynatten et al. 2023; Zaiko et al. 2023). However, this method has several limitations: (1) It can only detect a limited number of species at once. When screening for multiple invasive species, repeated testing is necessary, resulting in high costs. Repeated testing with qPCR requires additional sample material and increases costs, which can be a limiting factor for large-scale or long-term monitoring programmes; (2) it is challenging to detect invasive species in the early stages of invasion; (3) it is challenging to detect invasive species at trace DNA levels in the early stages of invasion. In such cases, nested PCR or digital PCR is typically needed, increased number of experimental steps and the reliance on more expensive equipment to the process; (4) its demand for comprehensive databases in the aquatic field is often incomplete, which leads to identification problems (Hindson et al. 2011; Whale et al. 2012; Wilcox et al. 2013; Wu et al. 2022).

Multiplex PCR (mPCR) technology enables the simultaneous amplification of multiple targets within a single PCR system (Chen et al. 2005; Zhong et al. 2020; Li et al. 2024). This not only reduces the amount of DNA required, but also enhances detection efficiency. mPCR has been widely applied to detect pathogenic bacteria (Altinok et al. 2008). In aquatic ecology, mPCR is commonly used

for identifying economically important species, distinguishing closely-related species and monitoring fish pathogen outbreaks (Zhong et al. 2020). The ability of mPCR to simultaneously amplify multiple target genes makes it a valuable tool for monitoring invasive species. For example, Wozney and Wilson (2017) utilised multiplex qPCR to investigate the presence of Asian Carp in the Great Lakes (Wozney and Wilson 2017). Amplicon sequencing not only offers high throughput, but also accurately distinguishes closely-related species, based on DNA sequence differences, enabling semi-quantitative monitoring through sequence abundance (Lundberg et al. 2013; Spilsberg et al. 2024). Due to its reliability and cost-effectiveness, amplicon sequencing has been widely utilised in bacterial community analysis and the development of whole-genome markers for animals and plants. The combination of amplicon sequencing and mPCR holds promise for advancing the screening of alien invasive species.

This study aims to develop an mPCR-based monitoring method for aquatic invasive species and apply it in the Pearl River Basin to achieve precise monitoring and minimise DNA requirements. This method allows for the simultaneous detection of multiple targets, significantly enhancing detection efficiency while reducing costs. It supports the efficient and accurate monitoring of aquatic invasive species, providing crucial technical support for future management and ecological conservation of these species.

Materials and methods

China common invasive species list and mPCR primers design

By reviewing the literature, official documents and open-source databases (Xu et al. 2004; Xu et al. 2012; Xiong et al. 2015; Wang et al. 2016; Xian et al. 2022), 46 key invasive species were selected, based on their harmfulness (Suppl. material 1: table S1). A total of 91 pairs of primers for mPCR amplification, including 31 pairs for 20 species, based on former studies (Suppl. material 1: table S2) and 60 pairs for 31 species newly designed from the mitochondrial genome (Suppl. material 1: table S3). Both D-loop and COI are considered to have high sequence variability and are suitable for designing species-specific primers (Ravago et al. 2002; Hebert et al. 2003; Aminisarteshnizi et al. 2024; Sumana et al. 2024). We verified all primers individually using the corresponding species' tissue DNA and found that all primers could be successfully amplified.

Invasive species standard plasmid design

Tissues of alien species were obtained through historical surveys and purchases. DNA was extracted from each invasive species (n = 46) using the Tissue DNA Kit (Egenomics, Nanjing, China), following the manufacturer's instructions. DNA concentration was measured using a Nanodrop spectrophotometer. The 91 pairs of invasive species-specific primers were used to amplify the tissue DNA and PCR products were obtained for each primer pair. Amplicons of the expected sizes were purified using an Agarose Gel DNA Purification Kit and then subcloned into the pMD-19T cloning vector (TaKaRa, Japan). Positive clones containing inserts of the expected size were sequenced using M13 primers. The PCR was conducted with an initial denaturation step at 94 °C for

5 minutes, followed by 35 cycles of denaturation at 94 °C for 10 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 30 seconds. A final extension step was performed at 72 °C for 10 minutes. Each 20 µl reaction mixture contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer, 1 U of Taq DNA polymerase and 5 ng of DNA.

Mix plasmid amplification

All plasmids were diluted to 100 ng/µl and 5 µl of each was mixed together. The upstream and downstream primers of the 91 species-specific primer pairs (0.02 µM each) were combined to create a primer mix. PCR amplification was then performed using the mixed plasmids as templates. The amplification system consisted of 10 µl of 2×Hieff NGS® HG Multiplex PCR Master Mix, 4 µl of multi-primer mix, 4 µl of ddH_2O and 2 µl of DNA template (100 ng/µl). PCR was carried out for 5 minutes at 94 °C, followed by 35 cycles of 94 °C for 10 seconds, 63 °C for 30 seconds and 72 °C for 30 seconds. A final extension step was performed at 72 °C for 10 minutes. PCR products were purified for sequencing.

Pearl river environmental DNA collection and extraction

The Pearl River Estuary, a critical aquatic ecosystem in southern China, is vulnerable to the introduction of invasive species that pose significant threats to biodiversity and ecosystem stability. In July 2021, thirty-two sampling sites (initially planned for 40 sites, but eight sites did not collect samples) were established in the Pearl River Estuary (Suppl. material 1: fig. S1). Three water samples were collected from each site using a 1 litre sampling bottle and then filtered through three mixed cellulose filter membranes (47 mm, 0.45 µm, Millipore, USA), respectively. After filtration, the membranes were transferred into 5 ml sterilised tubes and transported to the laboratory with dry ice. All samples were stored at -20 °C until DNA extraction. DNA was extracted from the filter membranes using the Water DNA Kit (Egenomics, Nanjing, China). DNA concentration and purity were measured by Qubit 2.0 and Nanodrop. The extracted DNA was stored at -20 °C. Filter 500 ml of ddH_2O as a negative control during the sampling, DNA extraction and PCR process. The plasmids for each species are for positive PCR controls.

mPCR and metabarcoding with environmental samples

Environmental sample mPCR amplification was performed in a total volume of 30 µl, consisting of 15 µl of 2×Hieff NGS® HG Multiplex PCR Master Mix, 6 µl of Primer Mix (0.01 µM), 8 µl of ddH_2O and 1 µl of DNA template. The reaction conditions of mPCR when amplifying plasmids and eDNA are consistent, except for the annealing temperature. The reaction conditions included pre-denaturation at 95 °C for 3 minutes, followed by 38 cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 30 s and extension at 72 °C for 30 seconds. A final extension was performed at 72 °C for 5 minutes, followed by storage at 4 °C.

Fish metabarcoding amplification of environmental sample using the 12S universal primers (Yang et al. 2023). The amplification reaction volume was 25 µl, consisting of 12 µl of 2×Taq Plus Master Mix II (Dye Plus) DNA polymerase (Novozymes, China), 2 µl of primer, 9 µl of ddH_2O and 2 µl of DNA template.

The reaction conditions included pre-denaturation at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 15 seconds, annealing at 62.4 °C for 20 seconds and extension at 72 °C for 20 seconds. A final extension was performed at 72 °C for 5 minutes, followed by storage at 4 °C.

High-throughput sequencing and bioinformatics analysis

The sequencing libraries of mPCR, metabarcoding and mixed plasmid were constructed using the same method. PCR amplification products of three repeated samples at each location were combined in equal volumes and purified using VAHTS DNA Clean Beads (Vazyme, Nanjing, China). The concentration of the purified products was measured using Qubit™ dsDNA HS Assay Kits (Invitrogen, USA). Sequencing adaptors were connected to purified DNA fragments using the Ion Xpress Plus Fragment Library Kit (Life Technologies, USA). Different libraries are assigned different indices when ligating the sequencing adapters. After assessing the quality and concentration of the sequencing libraries with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the libraries were diluted to 100 pM and subjected to high-throughput sequencing on an Ion Torrent S5 sequencer (Life Technologies, USA).

Low-quality (< Q20) and short reads (< 100 bp) were removed from the raw FASTQ files using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Yang et al. 2017). The FASTQ files were then converted to FASTA format using Mothur (Schloss et al. 2009). Sequences with no mismatches in tags and a maximum of two mismatches in primers were identified using the "split_libraries.py" script with the parameters "-w 50 -s 20 -M 2" and retained for further analysis (Zhang et al. 2020). Unique sequences were identified using the "derep_fulllength" script in Vsearch. Sequences were clustered into Operational Taxonomic Units (OTUs) using USEARCH. Species annotation was performed using a 97% similarity threshold with the previously constructed database and the Basic Local Alignment Search Tool (BLAST) (Zhang et al. 2023). All data analyses were conducted in R (version 4.1.2) using the Vegan package (Oksanen et al. 2022). All plots were generated using the ggplot2 package (Wickham et al. 2016). Pearson's correlation analysis was conducted to examine the relationships between invasive species detection by mPCR and metabarcoding.

Results

Validation of mPCR amplification using mixed standard plasmids

Of the 91 primer pairs tested, 70 successfully amplified target sequences, including 15 pairs adapted from published literature and 55 pairs newly designed in this study (Fig. 1a). The literature-derived primers primarily targeted the mitochondrial COI and Cytb gene regions, whereas the newly-designed primers primarily targeted the COI and D-loop regions (Fig. 1b). A total of 41 out of 46 aquatic invasive species were successfully detected, resulting in a species detection rate of 90%. Five species —*Pomacea canaliculata*, *Macrochelys temminckii*, *Oncorhynchus mykiss*, *Oreochromis mossambicus* and *Chelydra serpentina* – failed to amplify. Most amplicons were approximately 150 bp and the annealing temperatures of the primers were primarily around 65 °C (Fig. 1c, d).

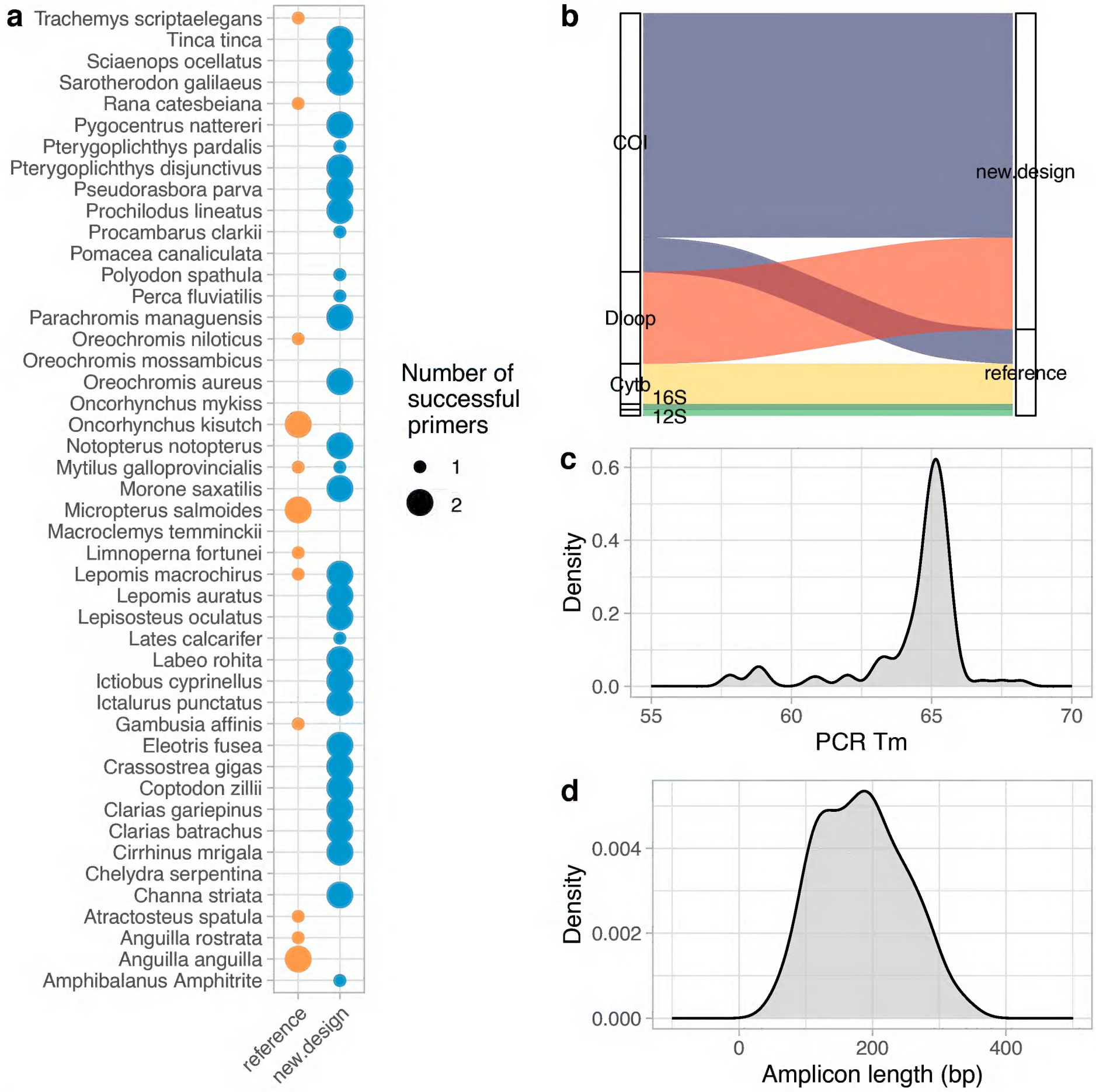

Figure 1. Multiplex PCR primers for common aquatic invasive species of China. **a** PCR primers for each species. **b** Genetic classification of PCR primer targets. **c** Distribution of primer annealing temperatures. **d** Distribution of PCR amplicon lengths.

Invasive species monitoring in the Pearl River Estuary using mPCR

In the mPCR assay, 33 primer pairs successfully amplified target seque-nces, detecting a total of 28 aquatic invasive species (Suppl. material 1: table S4). Amongst these, five fish species, including *Coptodon zillii*, *Cirrhinus mrigala*, *Oreochromis aureus*, *Sarotherodon galilaeus* and *Labeo rohita* were detected by two primer pairs (Fig. 2a). The invasive invertebrate species *Amphibalanus amphitrite* had the highest relative abundance in the Pearl River Basin, followed by *Tinca tinca*, *C. zillii* and *O. niloticus* (Fig. 2b). Of the 33 successfully amplified primer pairs, 23 targeted the COI region, accounting for 69.7%, while seven primer pairs targeted the D-loop region, accountin-g for 21.2%, those all being fishes (Fig. 2c). Several invasive species, including *Pygocentrus nattereri*,

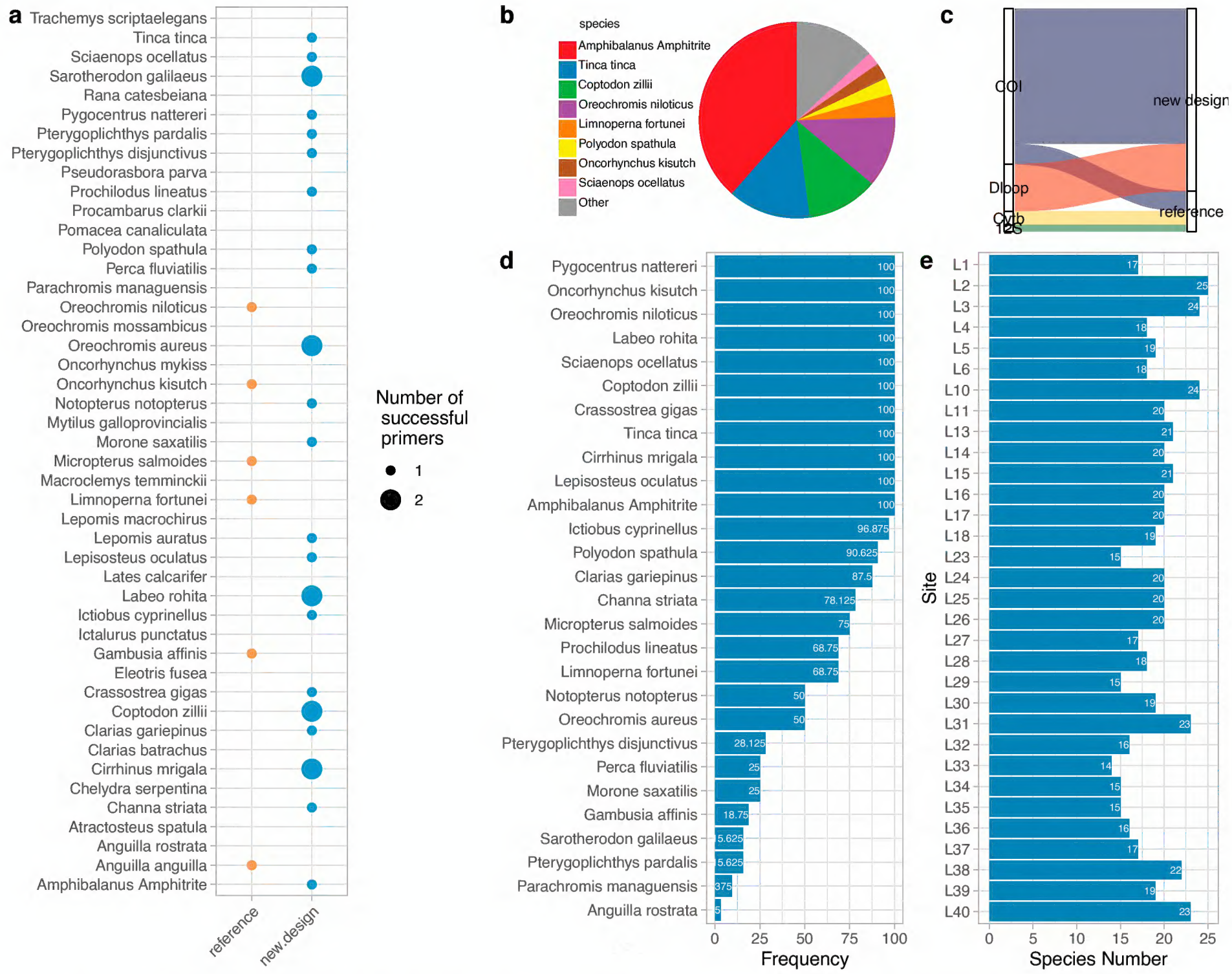

Figure 2. Invasive species monitoring in the Pearl River Estuary using mPCR. **a** Number of successful mPCR primers. **b** The sequence composition. **c** Classification of successful mPCR primers, based on target regions. **d** Detection rates of invasive species. **e** Number of invasive species detected at each site.

Oncorhynchus kisutch, *Oreochromis niloticus*, *Labeo rohita* and *Sciaenops ocellatus* were detected at all sampling sites (Fig. 2d). The number of invasive species detected at sites L2, L3, L10, L31, L38 and L40 was higher than that at other sites (species number > 22) (Fig. 2e).

At each site, species such as *A. Amphitrite*, *Tinca tinca* and *C. zillii*, all occupied a relatively high sequence abundance (Suppl. material 1: fig. S2). At estuarine sites (e.g. L26, L27, L37, L40), *A. amphitrite* exhibits a significantly dominant relative abundance; in inland river basins (e.g. L3, L13, L34), *O. niloticus* shows a significantly dominant relative abundance (Fig. 3).

Comparison of metabarcoding and mPCR

A total of 91 fish species were detected using 12S rRNA metabarcoding, including 11 invasive fish. All invasive fish detected by 12S rRNA metabarcoding could also be monitored by mPCR (Suppl. material 1: fig. S3). Compared with metabarcoding, mPCR can detect more species with fewer sequences (Fig. 4a, b). When only considering the 11 alien species jointly detected by the two

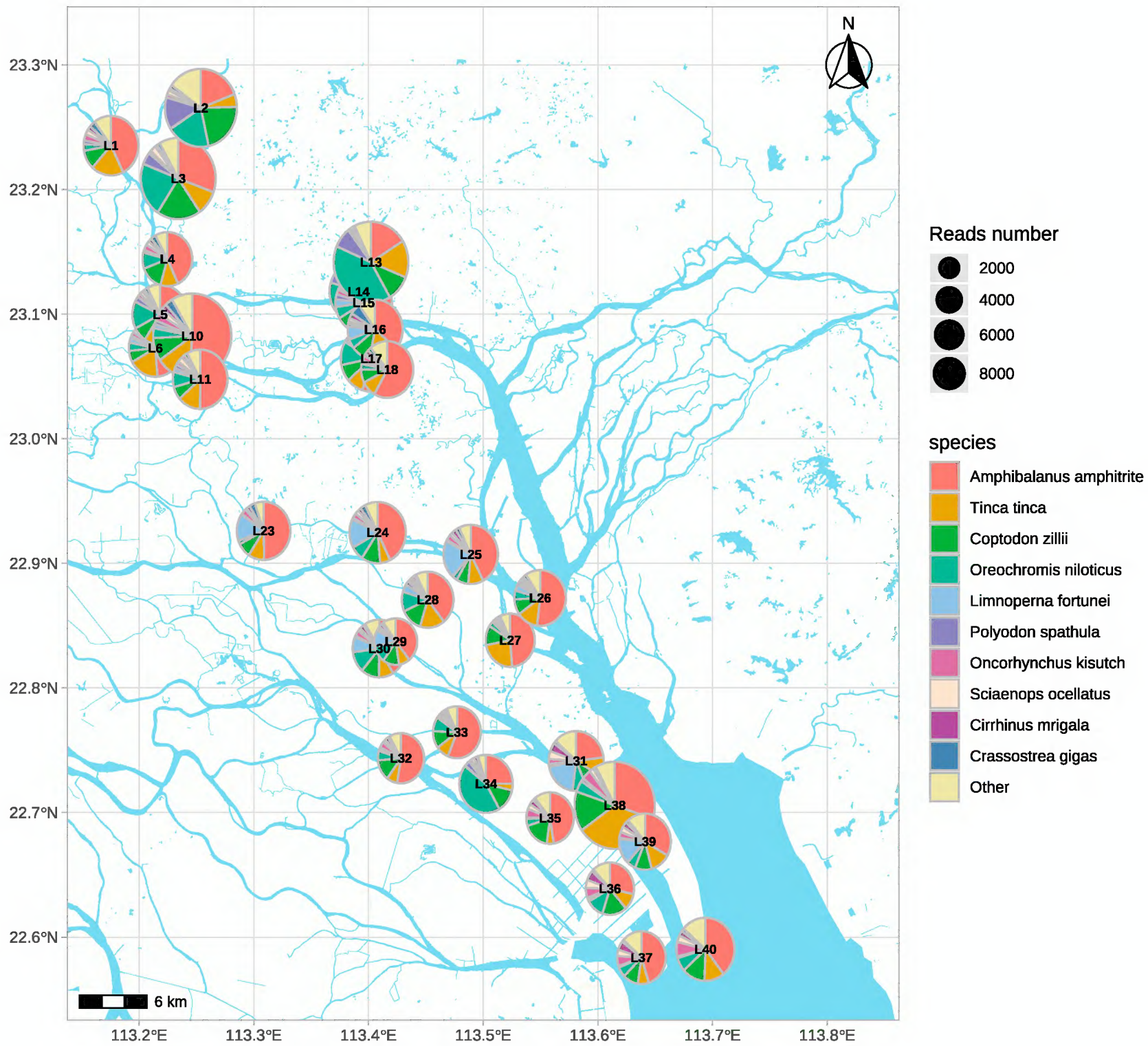

Figure 3. Top 10 invasive species in the Pearl River Estuary detected by mPCR.

methods, the 12S rRNA metabarcoding revealed that *O. niloticus* was the most abundant species across all sampling sites, followed by *C. zillii* and *O. aureus*. The mPCR method, on the other hand, showed the highest relative abundance of *C. zillii* and *O. niloticus*, followed by *O.aureus* and *C. mrigala* (Fig. 4c). The two methods jointly detected more sequences of the species at the upstream sites, such as L2, L3, L13 and L14 (Fig. 4d, f). For low-abundance species, such as *Pterygoplichthys pardalis*, which was clearly detected at sites L3 and L10 using 12S metabarcoding, it was rarely observed through mPCR (Fig. 4e, g).

A correlation analysis was conducted on 11 co-detected invasive species, revealing significant correlations in seven species (Fig. 5a) and no correlations in four species (Fig. 5b). Five species - *C. zillii*, *O. aureus*, *O. niloticus*, *P. pardalis* and *G. affinis* - exhibited “highly significant” correlations ($p < 0.01$). Two species, *C. striata* and *C. gariepinus*, showed “significant” correlations ($p < 0.05$). *C. zillii* and *O. niloticus* demonstrated particularly strong correlations with R^2-values exceeding 0.75, indicating high goodness-of-fit. A comparative analysis between metabarcoding and mPCR methodologies for detecting invasive species revealed a significant positive correlation ($p < 0.05$), as supported by the regression curve equation and R^2-value (Fig. 5c). This demonstrates strong methodological consistency in species detection capability between these two molecular approaches.

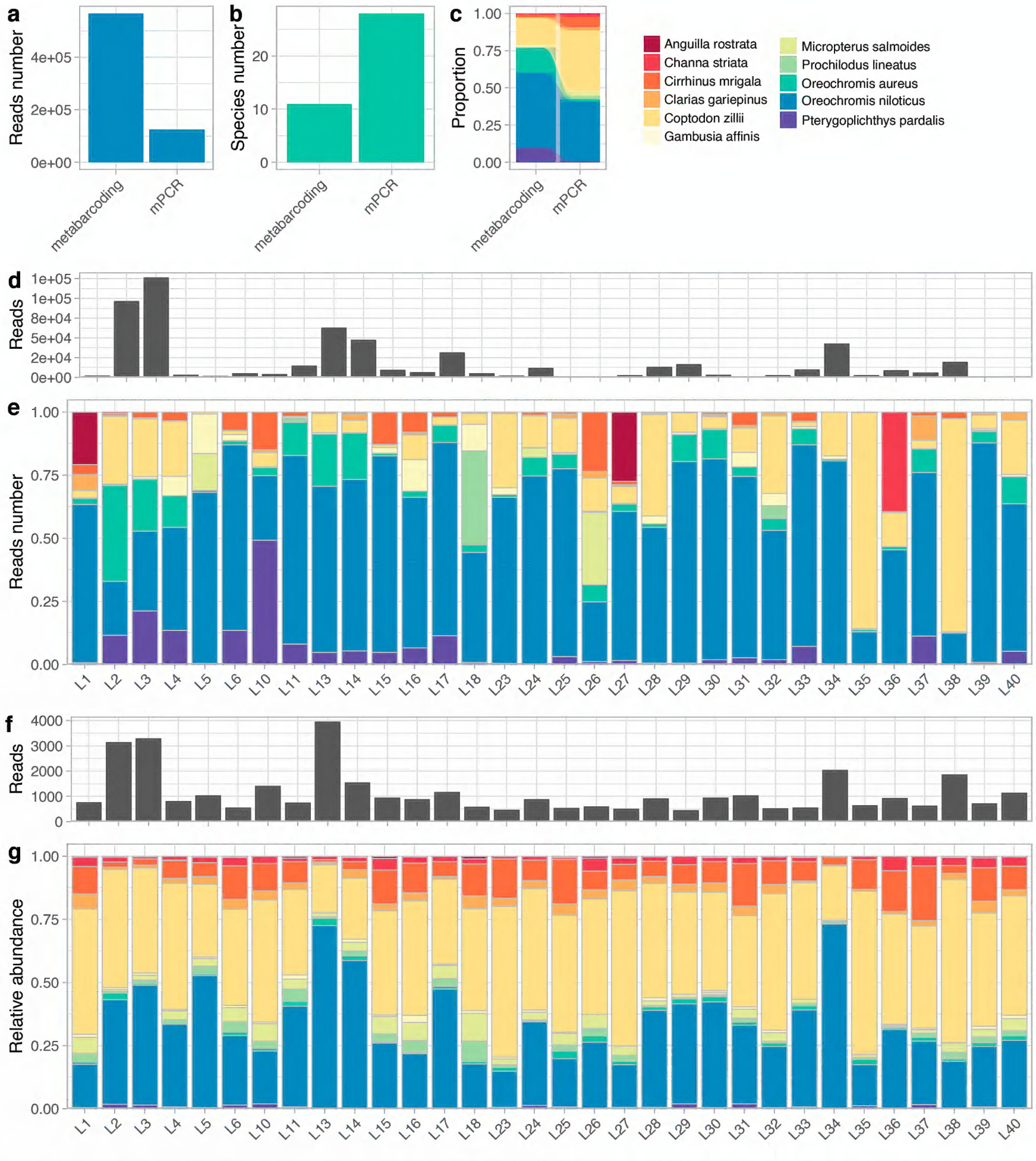

Figure 4. Comparison of invasive species that were detected by both metabarcoding and mPCR. **a** Read counts of all sequences. **b** All invasive species detected by metabarcoding and mPCR. **c** Relative abundances of invasive species that were detected by both methods. **d** Metabarcoding read counts of both detected species at each site. **e** Site-specific relative abundances of invasive species, based on metabarcoding. **f** mPCR read counts of both detected species at each site. **g** Site-specific relative abundances of detected species, based on mPCR.

Discussion

Accuracy of mPCR sequencing

All 91 pairs of species-specific primers successfully amplified in individual tests, but only 70 primer pairs yielded effective amplification in the mPCR system, with five species not detected. This outcome can be attributed to the inherent complexity of mPCR technology. First, primer interactions may reduce the amplification efficiency of some primers at high annealing temperatures. Unlike single

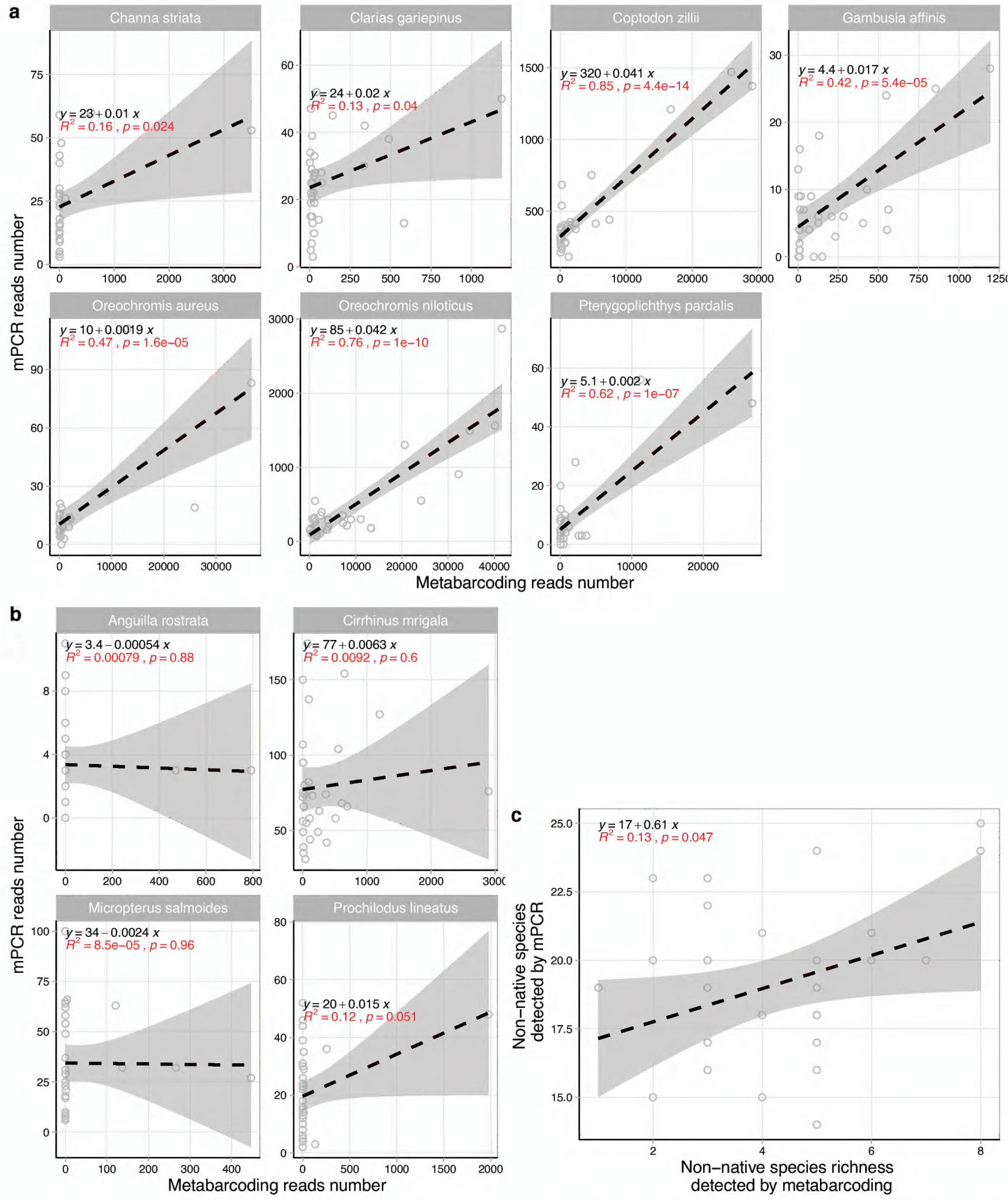
a
Channa striata
y = 23 + 0.01 x
R² = 0.16 , p = 0.024
Clarias gariepinus
y = 24 + 0.02 x
R² = 0.13 , p = 0.04
Coptodon zillii
y = 320 + 0.041 x
R² = 0.85 , p = 4.4e−14
Gambusia affinis
y = 4.4 + 0.017 x
R² = 0.42 , p = 5.4e−05
Oreochromis aureus
y = 10 + 0.0019 x
R² = 0.47 , p = 1.6e−05
Oreochromis niloticus
y = 85 + 0.042 x
R² = 0.76 , p = 1e−10
Pterygoplichthys pardalis
y = 5.1 + 0.002 x
R² = 0.62 , p = 1e−07
mPCR reads number
Metabarcoding reads number
b
Anguilla rostrata
y = 3.4 − 0.00054 x
R² = 0.00079 , p = 0.88
Cirrhinus mrigala
y = 77 + 0.0063 x
R² = 0.0092 , p = 0.6
Micropterus salmoides
y = 34 − 0.0024 x
R² = 8.5e−05 , p = 0.96
Prochilodus lineatus
y = 20 + 0.015 x
R² = 0.12 , p = 0.051
mPCR reads number
Metabarcoding reads number
c
y = 17 + 0.61 x
R² = 0.13 , p = 0.047
Non−native species detected by mPCR
Non−native species richness
detected by metabarcoding

Figure 5. Pearson correlation between invasive species detection by mPCR and metabarcoding. **a** Species with significant positive correlation. **b** Unrelated species; **c** Correlation between the invasive species richness detected by mPCR and metabarcoding.

PCR, mPCR requires a reduction in annealing temperature by 4–6 °C to allow for the co-amplification of multiple targets (He et al. 2009). Second, high-abundance non-target DNA may inhibit the amplification of low-abundance target DNA by competing for primer binding sites (Willson et al. 2013). Despite a few amplification failures, this study achieved a 90% species detection rate, reflecting the high overall reliability of the method. The undetected species could potentially be addressed by optimising primer concentrations or utilising a split-pool

amplification strategy. However, the current results are already adequate for large-scale screening needs. Our metabarcoding primers were designed primarily for fish taxa, which limited direct comparison for non-fish invasive species.

A preliminary biodiversity survey conducted in 2012 identified over 30 invasive species in the Pearl River Basin, including tilapia (*O. niloticus*, *O. aureus*), *Pangasius hypophthalmus*, *Clarias gariepinus*, *Gambusia affinis*, *Ictalurus punctatus*, *Cirrhinus mrigala*, *Labeo rohita* and *Micropteru+ s salmoides* (Gu et al. 2012). Over 50% of these species were detected in this study using eDNA mPCR sequencing. Tilapia species, such as *C. zillii* and *O. niloticus*, typically found in freshwater, showed significantly higher abundance at inland sampling sites (e.g. L2, L3, L13) compared to estuarine areas (e.g. L26, L27, and L40), aligning with their expected ecological distribution. Tilapia originated from freshwater sources in Africa and the Middle East (Renuhadevi et al. 2019). Without human intervention, the natural distribution of wild tilapia is mainly concentrated in inland areas. Although some tilapia can survive in waters with higher salinity, they are more adapted to freshwater environments. Artificial introduction is another important factor affecting the distribution of tilapia (Renuhadevi et al. 2019). Compared with coastal areas, inland areas are less affected by disastrous weather and are suitable for developing stable aquaculture (Esparza-Leal et al. 2010). Cultivating tilapia in inland areas can provide an important source of protein for local residents and promote local economic development (Yuan et al. 2017). *A. amphitrite*, a marine organism, was relatively abundant across all sites, likely due to its tendency to attach to ship hulls and buoys (Power et al. 2010), facilitating its transport from the Pearl River Estuary to inland waters. Species not detected, such as *C. serpentina*, are primarily found in the upstream tributaries of the Pearl River. Their lower population densities in estuarine areas may explain their absence in the study.

Consistency between mPCR and metabarcoding

The invasive species detected by mPCR were largely consistent with those identified through metabarcoding. All 11 invasive species detected by the metabarcoding approach were also successfully identified by the mPCR approach. Amongst these, high-abundance species such as *C. zillii*, *O. niloticus* and *O. aureus*, were stably detected by both methods, with a consistent trend in the distribution of their relative abundance. For low-abundance species, such as *P. pardalis*, which was clearly detected at sites L2 and L3 using the 12S method, it was rarely observed through mPCR. This difference can likely be attributed to primer design: mPCR primers are species-specific, enhancing sensitivity by overcoming the amplification bias of universal 12S primers (Bustin and Huggett 2017). A significant positive correlation was observed between the number of invasive species detected by both methods. Additionally, mPCR detected 17 invasive species not covered by 12S metabarcoding. This discrepancy is primarily due to methodological limitations, as universal 12S primers are designed to amplify vertebrate fish and cannot efficiently target crustaceans or molluscs. The 12S primers were initially designed for freshwater fish and are unable to accurately identify marine or brackish water species, such as *A. amphitrite*, *Crassostrea gigas*, *Morone saxatilis* and *Oncorhynchus kisutch*. In contrast, mPCR can effectively mitigate the sensitivity limitations inherent in metabarcoding and, in some cases, achieve sensitivity levels comparable to species-specific qPCR, dPCR or ddPCR assays.

In this study, mPCR detected 17 more species than metabarcoding. The enhanced detection capacity of mPCR is largely attributable to the use of species-specific primers, in contrast to the single universal primer pair employed in metabarcoding. The cost of mPCR is comparable to that of metabarcoding, lower than digital PCR and higher than qPCR. For long-term monitoring, another advantage of mPCR is its relatively low sample volume requirement, a benefit that becomes increasingly pronounced as the number of target alien species rises. Amongst the 11 tilapia species co-detected, the majority of sequences obtained by mPCR were concentrated in two species, with a relatively strong correlation between the methods. For the remaining species, both methods yielded comparatively few sequences, which may explain the weaker correlations observed. This suggests that eDNA detection results are more reliable when sequence counts are higher. The advantages of mPCR include its high efficiency, high specificity, lower sequencing depth requirements and lower costs. It is especially suitable for targeted screening of known high-risk invasive species (Altinok et al. 2008). In contrast, 12S metabarcoding is better suited for broad-spectrum surveys of fish communities, though it has limitations in detecting non-fish taxa and low-abundance species. However, the situation varies across taxa or when different methods are applied to the same group of organisms. For example, in studies of fish, crustaceans or molluscs, a dual-marker strategy is often employed to compensate for taxa that a single marker fails to detect. While this approach can overcome the limitations of single-marker analyses, it also entails higher costs and longer processing times. The two methods are complementary and together provide multi-level technical support for invasive species monitoring.

Ecological impacts and prevention of invasive species

In the Pearl River Basin, *O. aureus*, *O. niloticus* and *O. mossambicus* have been identified as the dominant invasive species. Tilapia were primarily introduced for aquaculture purposes and, with the expansion of the aquaculture industry and the increasing frequency of species introductions, tilapia have spread through both human-mediated dispersal and natural migration within river ecosystems (Vicente and Fonseca-Alves 2013; Xiong et al. 2022; Yongo et al. 2023). They have now escaped into natural waterbodies and become one of the most widely distributed and heavily farmed aquatic invasive species in China (Sun et al. 2018; Forneck et al. 2020). Aquatic invasive species often functionally differ from the native components of the ecosystems they invade, exerting significant negative impacts on the biodiversity of aquatic organisms, particularly macrophytes, zooplankton and fish (Gallardo et al. 2015) Enclosure experiments have demonstrated that *O. niloticus* can increase nutrient loading in waterbodies, leading to higher phytoplankton biomass and reduced water transparency, with a more pronounced top-down impact on the aquatic ecosystem than bottom-up effects (Liu et al. 2008). Furthermore, as omnivorous fish, tilapia not only promote phytoplankton growth in shallow lakes by increasing nutrient availability, but also inhibit the growth of benthic algae through sediment re-suspension, thereby increasing water turbidity (Zhang et al. 2016; Liu et al. 2023).

The invasion of tilapia in the Pearl River may have a long-term negative impact on local species diversity and ecological balance. Therefore, more effective management strategies should be explored to minimise its spread and

ecological impact in natural waterbodies. For example, Sunarto et al. (2022) found that the use of tilapia lake virus (TiLV) leads to high mortality rates of wild and farmed tilapia, but does not affect the mortality of other species and relies on water as a means of dispersal, which has a high potential for biocontrol of invasive tilapia. Based on the high-abundance areas of tilapia identified in this study (e.g. *O. aureus* accounting for 44% at site L34), it is recommended that the implementation of ecological interception measures be prioritised in these areas to reduce nutrient input. Compared to traditional morphological or single-locus PCR methods, this study offers technical support for the early detection of aquatic alien invasive species, particularly through identifying species with unexpectedly high local abundance. For example, *A. amphitrite* and *T. tinca* showed high relative abundance in sites such as L1, which may indicate recent ecological shifts or introduction pathways. These findings offer valuable insights for targeted management and control strategies (Gallardo et al. 2016).

Multiplex PCR offers several advantages for detecting invasive species, including convenience, efficiency and low cost. The results of this study indicated that all alien species detected through metabarcoding could also be identified using mPCR, with a significant positive correlation between the abundance of commonly detected species by both methods. While metabarcoding is more suitable for monitoring high-abundance alien species due to its species-specificity, mPCR excels at accurately detecting low-abundance species, making it particularly advantageous in scenarios requiring precise identification. These findings highlight the strengths and advancements of mPCR in monitoring aquatic invasive species and underscore its important role in early warning systems for such species. In conclusion, the mPCR detection system provides valuable technical support for the early detection and monitoring of invasive species. This method can be further optimised for monitoring rare or endangered species, contributing to more effective conservation efforts and supporting global biodiversity protection initiatives.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

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Data availability

The high-throughput sequencing raw data are available in the NCBI BioProject (PRJNA1307428) and in the Sequence Read Archive repository under accession numbers from SRR50665889 to SRR50665926. The custom code used for statistical analysis and generating all figures in this study has been deposited in the GitHub repository at https://github.com/jhyang888/mPCR and is publicly available as of the date of publication.

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Supplementary material 1

Invasive Species Monitoring Based on eDNA Multiplex PCR Sequencing

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